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Address for correspondence: Florent Valour, Department of Infectious Diseases, Hospices Civils de Lyon, 103 Grande-Rue de la Croix-Rousse, 69004 Lyon, France; email: florent.valour@chu-lyon.fr

Loss of 89K Pathogenicity Island in Epidemic *Streptococcus suis*, China

Xiaolu Shi,¹ Huiyan Ye,¹ Jun Wang, Zhencui Li, Jingzhong Wang, Baoshan Chen, Ronghui Wen, Qinghua Hu, Youjun Feng

Author affiliations: Zhejiang University School of Medicine, Hangzhou, China (X. Shi, H. Ye, J. Wang, Z. Li, Y. Feng); Shenzhen Centre for Disease Control and Prevention, Shenzhen City, China (X. Shi, J. Wang, Q. Hu); Guangxi University, Nanning City, China (H. Ye, J. Wang, B. Chen, R. Wen)

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To the Editor: *Streptococcus suis* serotype 2 (SS2) is a previously neglected, newly emerging human pathogen that causes occupational and opportunistic infections (1,2). Outbreaks of fatal human SS2 infections in China, featuring streptococcal toxic shock syndrome, in 2005 seriously challenged global public health (3–5). The epidemic strain is unusual in that it contains a unique 89-kb (89K) pathogenicity island (PAI) (3,6,7). We observed the loss of genes from the 89K PAI in sporadic cases in southern China in 2007, implying the dynamic evolution of this PAI (8). Therefore, 89K PAI might be able to be used to monitor prevalent strains of *S. suis* in China (8).

We report 10 recurrent cases of human *S. suis* infections during 2008–2015 in southern China. Most of the hospitalized patients were male workers in close contact with pigs, pork products, or both. These patients typically exhibited clinical syndromes of meningitis, including headache, coma, vomiting, and fever. The bacterial strains acquired from humans were as follows: 2 isolates in 2008 (Stre08001 and Stre08002), 2 in 2009 (Stre09001 and Stre09002), 2 in 2011 (Stre11001 and Stre11002), 3 in 2013 (Stre13002,

Stre13003, and Stre13004), and 1 in 2015 (Stre15001). Microbial and molecular assays proved that these clinical isolates were *S. suis* (online Technical Appendix Figures 1, 2, <http://wwwnc.cdc.gov/EID/article/22/6/15-2010-Techapp1.pdf>). Multiplex PCR-based molecular determination (16S rDNA, *mrp*, *epf*, and *cps-2j*) suggested that all strains except Stre13002 were SS2 (online Technical Appendix Figure 2) (3). To determine whether these clinical isolates derived from the same Chinese epidemic clone 05ZYH33, we sequenced an array of virulence factor-encoding genes, as well as the 16S rDNA genes. Phylogenetic trees indicated that all 10 clinical strains are classified into the same subclade as that of the strain 05ZYH33 (online Technical Appendix Figure 2).

Subsequent analyses by pulsed-field gel electrophoresis revealed that genotypes are diverse among these clinical strains, which can be roughly divided into 6 groups (online Technical Appendix Figure 3). Given that the Sao surface antigen protein possesses 3 allelic variants (Sao-L [670 aa], Sao-M [580 aa], and Sao-S [489/490 aa]) (9), we thus assayed it with these clinical strains. Unexpectedly, we found 2 more new allelic variants, referred to as Sao-L1 (640 aa) and Sao-L2 (611 aa). Except for the strain BM407, which is a Chinese epidemic SS2 encoding Sao-L1, a version 30 residues shorter than Sao-L, 8 of the 10 clinical *S. suis* isolates consistently had the same new form of Sao protein, Sao-L2 (611 aa) (online Technical Appendix Figure 4).

Because 89K PAI is in dynamic evolution, determining whether it remains present in clinical strains is of interest. As previously designed (online Technical Appendix Table 1), a specific pair of boundary primers (1/6) was applied for PCR-based detection of the 89K PAI (Figure, panel A, <http://wwwnc.cdc.gov/EID/article/22/6/15-2010-F1.htm>). In principle, the PCR-positive result suggests the absence of 89K PAI, whereas the PCR-negative result indicates the presence of 89K PAI (6,8). Unlike the epidemic strain 05ZYH33 that has the 89K PAI, 9 of the 10 clinical strains examined (Stre08001, Stre08002, Stre09001, Stre09002, Stre11001, Stre11002, Stre13002, Stre13003, and Stre13004) were unexpectedly found to be PCR positive for the unique 1/6 DNA fragment with expected size of ≈1.5 kb (Figure, panel B). This finding indicates that the 89K PAI is lost in these 9 clinical strains. We saw similar scenarios in the subsequent PCRs for other inner genes/DNA fragments (943 and 944 [10]; 1/2, 3/4, and 5/6 [8]) inside of 89K PAI (Figure, panel C). Further DNA sequencing of the 1/6 PCR product showed that it matches well with the 2 boundary regions neighboring the 89K PAI, validating the loss of 89K PAI in these 9 clinical isolates (Figure, panel D). In contrast, the strain Stre15001 behaved similarly to that of the 05ZYH33 containing the 89K PAI, in that both are PCR positive for the 4 amplicons

¹These authors contributed equally to this article.

of 1/2, 5/6, 943, and 944 but PCR negative for the 1/6 amplicon (Figure, panels B–D). The only minor difference between strains Stre15001 and 05ZYH33 lay in the 3/4 amplicon (Figure, panel C). Clearly the 3/4 DNA fragment is present in the 89K PAI from strain 05ZYH33 but not in the counterpart of the strain Stre15001 (Figure, panel C); that is, strain Stre15001 carries a variant of 89K PAI lacking (at least part of, if not all) the 3/4 DNA fragment. In terms of 89K PAI (and pulsed-field gel electrophoresis/Sao protein), we propose that a heterogeneous SS2 population is circulating in China. Also, we observe that the differentiation of bacterial virulence is related to the clinical strains using the infection model of Balb/c mice (online Technical Appendix Figure 5).

In summary, the loss of 89K PAI might highlight the emergence of an epidemic SS2 population. This population appears to have genetic heterogeneity that is undergoing evolution in an adaption to some selection pressure from the environment, host restriction, or both.

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Y.F. and Q.H. designed this project; X.S., H.Y., J.W., and Z.L. performed experiments and analyzed the data; B.C. and R.W. contributed reagents and tools; Y.F. wrote the article.

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Address for correspondence: Youjun Feng, Department of Medical Microbiology and Parasitology, Zhejiang University School of Medicine, Hangzhou, Zhejiang 310058, China; email: fengyj@zju.edu.cn; or Qinghua Hu, Shenzhen Centre for Disease Control and Prevention, Shenzhen City, Guangdong 518055, China; email: huqinghua03@163.com

Next-Generation Sequencing of *Mycobacterium tuberculosis*

Igor Mokrousov, Ekaterina Chernyaeva, Anna Vyazovaya, Viacheslav Sinkov, Viacheslav Zhuravlev, Olga Narvskaya

Author affiliations: St. Petersburg Pasteur Institute, St. Petersburg, Russia (I. Mokrousov, A. Vyazovaya, O. Narvskaya); St. Petersburg State University, St. Petersburg (E. Chernyaeva); Research Institute of Phthisiopulmonology, St. Petersburg (E. Chernyaeva, V. Zhuravlev, O. Narvskaya); Scientific Center of Family Health and Reproductive Problems, Irkutsk, Russia (V. Sinkov)

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To the Editor: Next-generation sequencing (NGS) technology is becoming more affordable and is increasingly being widely used for high-resolution molecular epidemiology of tuberculosis. Using an example of the emerging multidrug-resistant strain of *Mycobacterium tuberculosis*, we showed the value of informed understanding when in silico prediction from NGS data achieved with available bioinformatics tools is placed within the context of the existing genotyping framework.

Spoligotyping is a classical method of *M. tuberculosis* genotyping, and the SITVIT_WEB database contains data on 7,105 spoligotype patterns of 58,180 isolates from 153 countries (http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE). Spoligotyping targets a variation of the DR/CRISPR locus, whose evolution in *M. tuberculosis* occurs through deletion of single or multiple spacers. By

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Technical Appendix

Technical Appendix Table 1. Primers carried for PCR-based molecular identification of epidemic *Streptococcus suis*, China

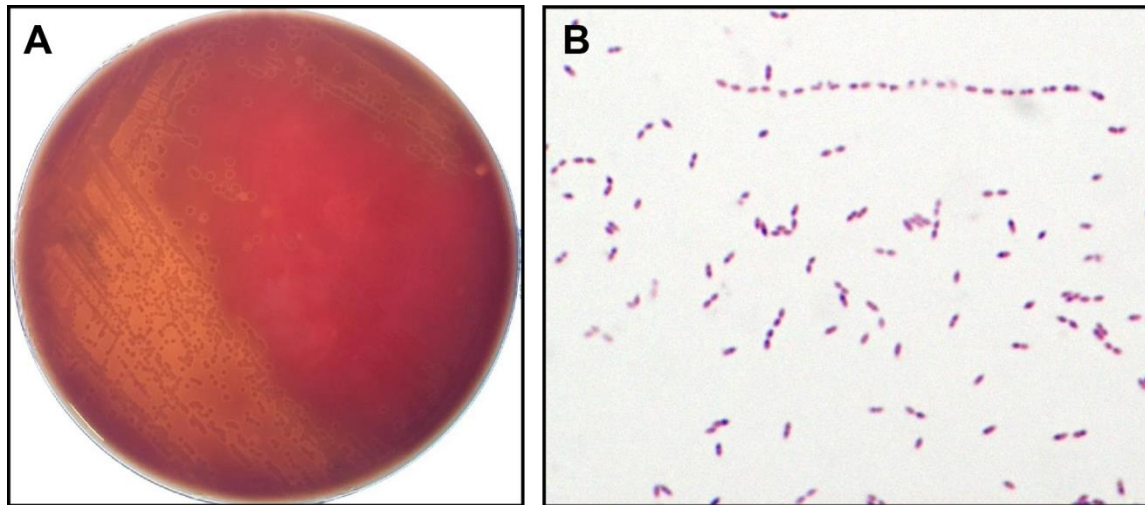
Primer	Primer sequence	Reference	Target gene (kb)
1	5'-CACGCATCTCGTAGAGTTTGAC-3'	(1,2)	1/2 (≈2.1)
2	5'-AGATTGCGAGGCTTTTAGATTG-3'		
3	5'-TCGCCACTATGGTATCTGCTTA-3'	(1,2)	3/4 (≈0.7)
4	5'-GATTGTGGACCATGCTGTTTAG-3'		
5	5'-ATAAATAGCCCCATCCTCATCA-3'	(1,2)	5/6 (≈0.8)
6	5'-GCGTAGCTGCTTAGTGCTACAA-3'		1/6 (≈1.5)
943-F	5'-TTGAAAATTTTATTAATAGATG ATC-3'	(2)	'943' (≈0.6)
943-R	5'-TTACTTAATATATCCTAACTTCTG-3'		
944-F	5'-TTGTTTTTTTCAA AAGTTACAGAC-3'	(2)	'944' (≈1.2)
944-R	5'-TTATCTATTAATAAAATTTTCAATTGCC-3'		
16S-F	5'-CAGTATTACCGCATGGTAGATAT-3'	(2)	16S rDNA (≈0.3)
16S-R	5'-GTAAGATACCGTCAAGTGAGAA-3'		
mrp-F	5'-GGTATACCTTGCTGGTACCGTTC-3'	(2)	mrp (≈0.6)
mrp-R	5'-AGTCTCTACAGCTGTAGCTGG-3'		
epf-F	5'-ACAAAGGCGTAGGTTCAATC-3'	(2)	epf (≈0.3)
epf-R	5'-CGGCATCAAGAATGTCTTTG-3'		
sao-F	5'-ATGAATACTAAGAAATGGAG-3'	(3)	sao-M (≈1.8)
sao-R	5'-TTATAATTTACGTTTACGTGT-3'		
2J-F	5'-TGATAGTGATTTGTCGGGAGGG-3'	(2,4)	cps-2J (≈0.5)
2J-R	5'-GAGTATCTAAAGAATGCCTATTG-3'		
sly-F	5'-GCAGATTCCAAACAAGAT-3'	(2)	Suilysin (≈1.4)
sly-R	5'-CTCTATCACCTCATCCGC-3'		

*The PCR experiments were conducted according to the primer combinations below (1&2, 3&4, 5&6, and 1&6).

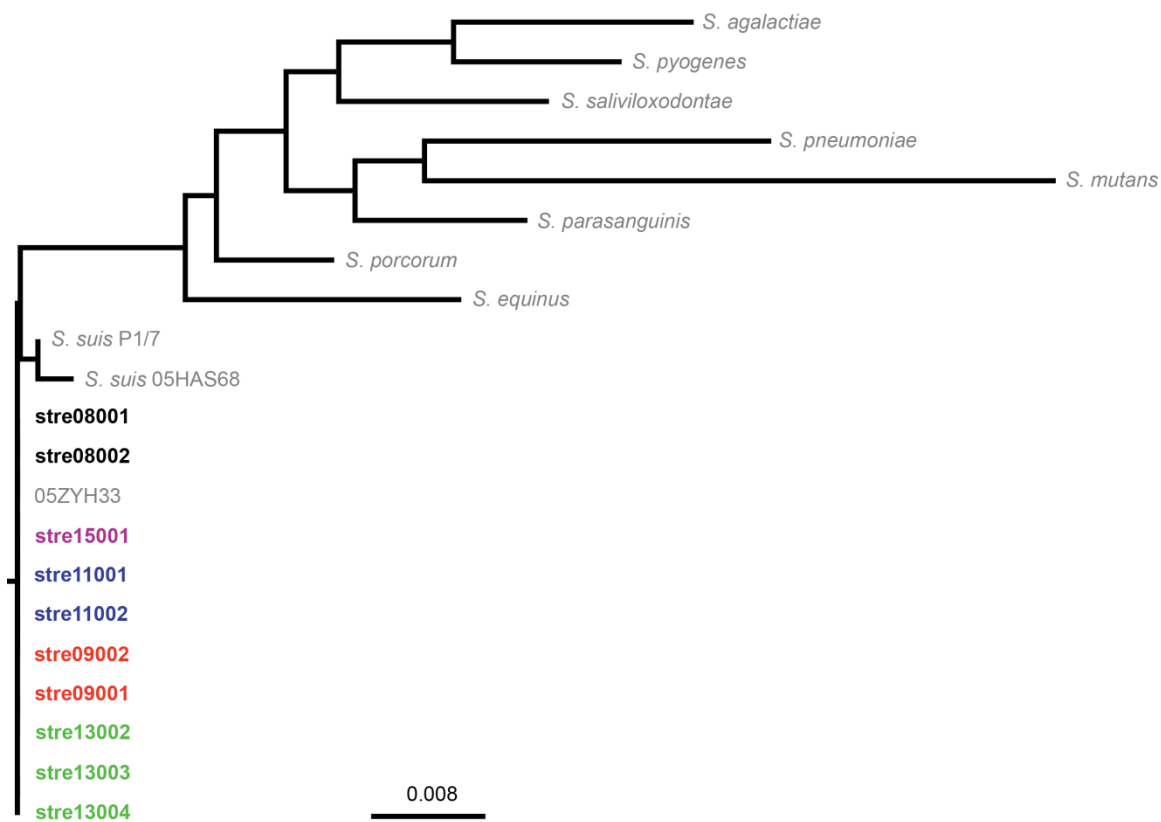
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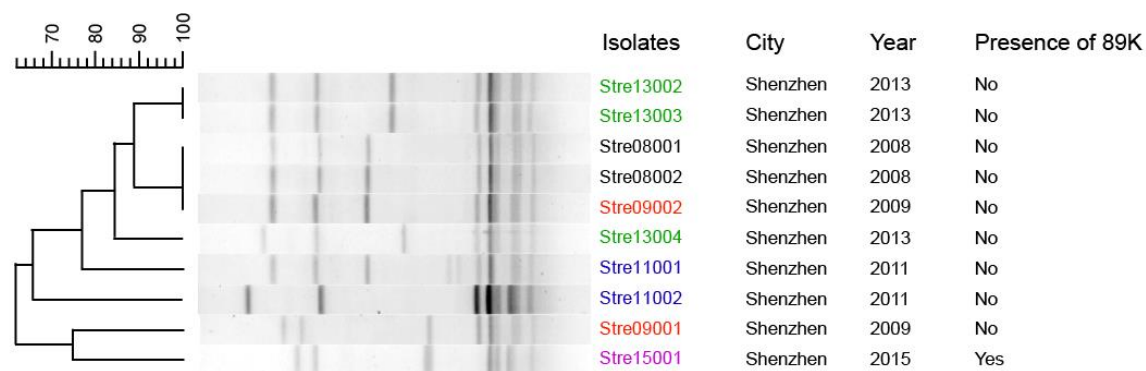
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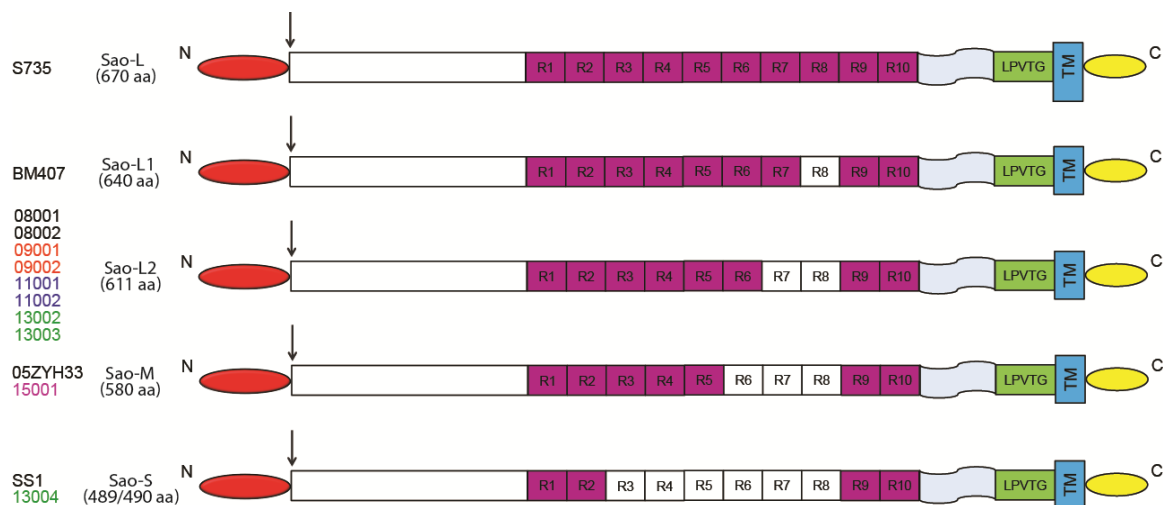
Technical Appendix Figure 1. Microbial characterization of the representative isolate of human *Streptococcus suis*, China. A) Colony phenotype of the isolated *S. suis* growing on the THB agar plate supplementing 5% sheep blood Yellow indicates the hemolytic activity. B) Light microscopic analyses of the *S. suis* cultures after Gram staining.



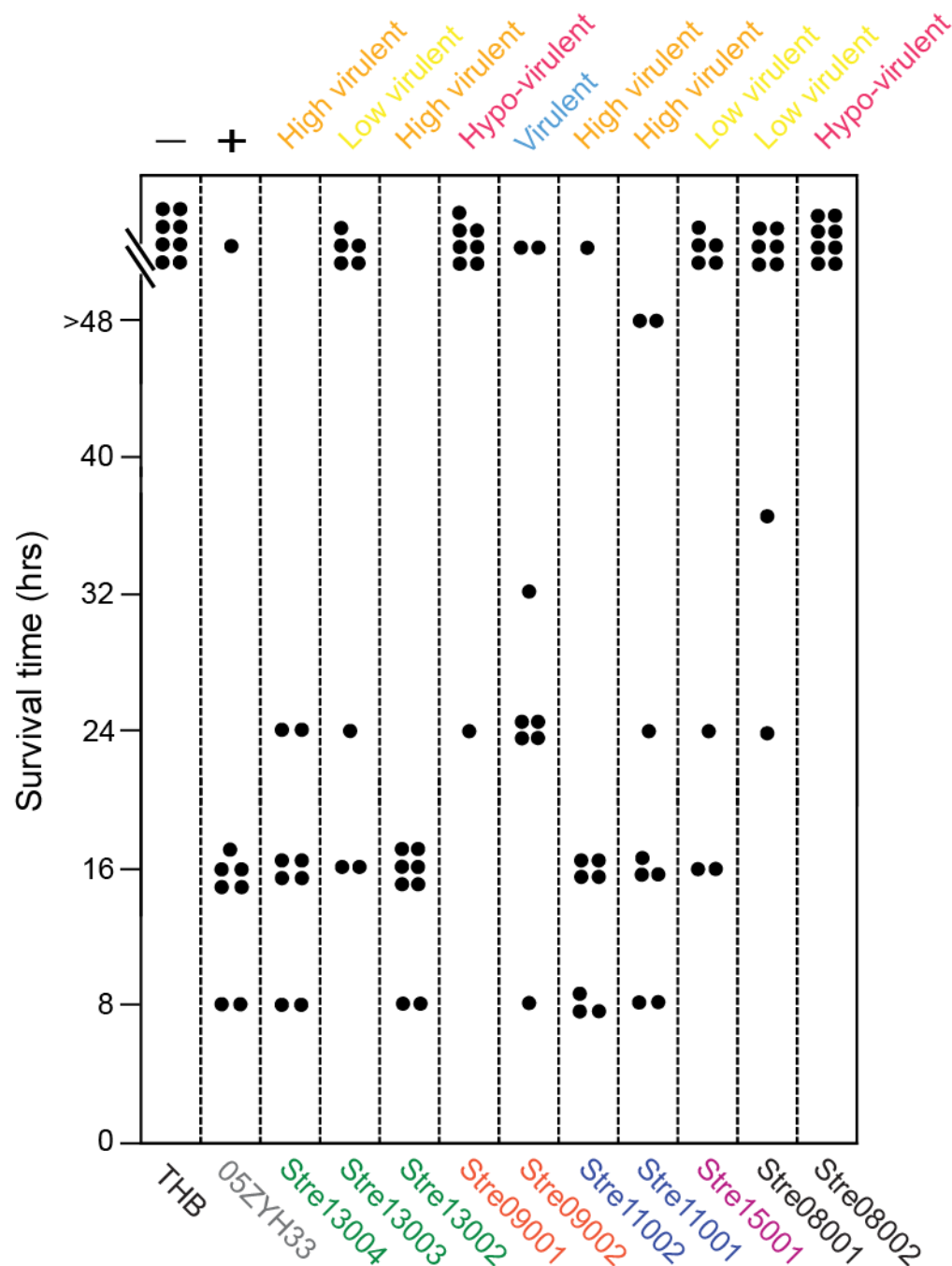
Technical Appendix Figure 2. 16S rDNA-based phylogeny of the newly isolated *Streptococcus suis* strains. The phylogenetic tree was constructed by using the program of ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2>), and the final output was given with the software of FigTree (<http://tree.bio.ed.ac.uk/software/figtree>). The international reference strain is *S. suis* 2 is P1/7, and the 2 known Chinese *S. suis* serotype 2 strains included 05ZYH33, an epidemic Chinese virulent strain and an avirulent strain 05HAS68. The 10 strains identified from Shenzhen City are in bold and color.



Technical Appendix Figure 3. PFGE analyses for the newly isolated Chinese *Streptococcus suis* strains. The genomic DNA of 10 bacterial species were isolated and digested with SmaI in gel. Clustering of PFGE patterns was conducted by an unweighted group with arithmetic averaging. The dendrogram of PFGE profiles was drawn using BioNumerics software 3.0 (Applied Maths, Austin, TX, USA). 89K is an abbreviation for a 89-kb pathogenicity island. PFGE, pulsed-field gel electrophoresis.



Technical Appendix Figure 4. Heterogeneity of Sao surface protein from the *Streptococcus suis* populations. Linear diagram for the 5 types of Sao proteins is given. The vertical arrow indicates the predicted cleavage site by signal peptidase. The ellipses in red and yellow depict the hydrophobic region at the N-terminus and the charged amino acids of the C-terminal tail. TM indicates the putative transmembrane region at the C-terminus. Curved grey boxes indicate the possible cell wall-associated regions. White boxes indicate deletion of repeated regions. Five types of Sao proteins were attributed to *S. suis* population, adding 2 more new forms (Sao-L1 and Sao-L2) to the former scenarios (Sao-L, Sao-M, and Sao-S). Sao-L: SaoO protein of 670 aa long; Sao-L1: Sao protein of 640 aa long; Sao-L2: Sao protein of 611 aa long; Sao-M: Sao protein of 580 aa long; Sao-S: Sao protein of 489/490 aa long. Multiple-sequence alignment of Sao protein suggested that the repeat regions are present in the C-terminus, but the number of repeat regions varies. R1, R2, R3 . . . R10: Repeated region1, 2, 3 . . . 10 in the C-terminus of Sao. S735: Holland isolate of *S. suis* 2 (Sao-L); BM407: A Chinese virulent strain of *S. suis* 2; SS1: Holland strain 5428 of *S. suis* 1 (Sao-S); 05ZYH33: an epidemic strain isolated from human *S. suis* 2 outbreak in China, 2005 (Sao-M). All the other *S. suis* strains reported here are numbered/indicated in color.



Technical Appendix Figure 5. Virulence differentiation the Chinese *Streptococcus suis* population in the mice-based infection model. In total, 12 groups of Balb/c mice (8 mice each group) were subjected to intraperitoneal (I.P.) injection with *S. suis* strains at a dose of 10^9 CFU/mouse. The wild type/virulent strain of *S. suis*, 05ZYH33, acts as positive control (+); THB indicates blank control (--). The 10 strains of clinically isolated *S. suis* included Stre08001, Stre08002, Stre09001, Stre09002, Stre11001, Stre11002, Stre13002, Stre13003, Stre13004, and Stre15001, respectively. Survival time (hrs) of individual mouse is monitored during the entire period of infection. Here, the virulence of these newly collected clinical *S. suis*

isolates is differentiated into 4 groups: high virulent, virulent, low virulent, and hypovirulent (nonvirulent). The criteria for bacterial virulence differentiation are as follows: 1) high virulence for 7--8 deaths among the 8 infected mice (7/8--8/8); 2) virulence for no less than 6 deaths among the 8 challenged mice (6/8); 3) low virulence, 2--3 deaths among the 8 mice after -infection (2/8--3/8), and 4) hypovirulence (nonvirulence) decodes no more than 1 death among the 8 mice after the challenge (0/8--1/8).